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Review Article

The Consequences and Challenges Associated with Amphibian Toxicology Regarding Pesticides

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ABSTRACT

Amphibian populations worldwide are experiencing a decline due to a combination of abiotic and biotic factors. Climate change, habitat loss, pollution, and disease outbreaks all contribute to this decline. Many amphibian species are listed as vulnerable or near extinct (43% of the species described nowadays) on the IUCN Red List. Anthropogenic contaminants, particularly pesticides, can be incredibly harmful to these populations. Pesticides can come from different sources, in particular from agriculture. Contamination of animals can occur through ingestion of contaminated feed, air, drift, secondary poisoning, spillage into local water bodies, contaminated plants and sediments, or groundwater contamination. Higher concentrations of pesticides in the environment can have acute toxic effects with high mortality rates, or long-term exposure can lead to reproductive abnormalities, infertility, and malformations. Several papers have implicated pesticides in the amphibian population decline. The primary objective of the research was to establish a link between the use of pesticides and the decline of amphibian populations, focusing on documented cases in the wild where these chemicals have been identified as the primary cause of mortality among these species and assessing their broader ecological impacts. Additionally, the study aimed to highlight the main challenges encountered in conducting ecotoxicological research on amphibians and to explore potential avenues for future research and mitigation efforts.

1. Introduction

Amphibians represent the most threatened group of vertebrates[1]. They have existed for 350 million years and are distinguished by their variety of species. The Amphibia Class comprises the Orders Caudata (salamanders), Anura (frogs and toads), and Gymnophiona (caecilians) [1-3].

Since 1980, there has been a significant decrease in amphibian populations worldwide[2]. More than 160 amphibian species have become extinct in the last decades[4]. The recent global assessment of amphibians reveals that approximately 43% of known amphibian species are declining, with around 32% facing a severe threat of extinction. Furthermore, a significant portion, approximately 22.5%, lacks sufficient data to determine their status definitively[4]. According to the data available in the 2004 IUCN Red List 5, 53% of amphibian species declined in Western Europe, 54% in North America, 60% in South America, and 70% in Australia and New Zealand[6].

The global decline in amphibian populations is attributed to countless issues, including habitat loss, climate change, environmental stress directly or indirectly by human beings, over-exploitation, diseases, and pollution[1]. Nevertheless, the relative importance and synergies among various causes remains limited[1].

Amphibians are an essential component of the ecosystem. They can be found almost everywhere, from forests, swamps, meadowlands, ditches, suburbia, and urban areas to agricultural lands[1]. Amphibians have an important role in the ecosystem health and provide important services[7]. Adult amphibians feed on insects of a large number of insects and other invertebrates, protecting crops from pests and eliminating vectors carriers of disease[7]. They also have the potential to pollinate and seed dispersal since they consume insects (e.g., butterflies, beetles) that carry pollen in their exoskeleton, and even some species of tropical amphibians (e.g., *Xenophile truncata*) consume fruits[8]. Amphibians are a source of food

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for several species, including humans and biomedicines. For instance, *Latoria caerulea* produces a skin secretion known as caerinthat, which can eliminate plasmodium, nematodes, and viruses, and acts as an anticancer[9]. Poisonous amphibians are donors of biologically active substances, including medicinal raw materials, to produce drugs[10]. For example, in the skin of poisonous of the poisonous frog, Bombina bombina is a peptide denominated Bombesin that indicates a high affinity for gastrin-releasing peptide receptors[10].

The most important role of amphibians is as a biological indicator of ecosystem health. They are easily affected by any change that occurs in the environment[6]. Through their skin, they can absorb gases and water that can contain pollutants. Some species of larvae are filters, so when they clean up the freshwater, they can accumulate any toxic components in the water. A reduction in amphibian numbers can have serious consequences[6,11]. Their position in the food chain, semi-permeable skin, and the development of eggs and larvae in the water are some characteristics that make them excellent environmental sentinels[6].

Many factors that contribute to the decline of amphibians are human-induced, such as pollutants in special agrochemical products[12]. Various types of pesticides and their residues exist in various aquatic and terrestrial habitats at different concentrations and mixtures[13]. In addition, some pesticides are non-biodegradable, having a long half-life and persisting in the environment. Moreover, they are highly lipophilic and can accumulate in the food chain[13]. These features make them very difficult to remove, and their adverse effects can be seen many years after their use has been banned[13–15].

The transformation of the habitats to urban and agricultural uses had many negative effects on climate, soil fertility, biogeochemical cycles, land use, and diversity[16]. The fragmentation of habitat also has an impact on amphibian populations. Amphibians depend on the quality of aquatic habitats and surrounding landscaping for reproduction and development[16]. Anthropogenic transformation of the landscape is very heterogeneous, therefore, imposing new adaptation challenges to amphibian populations. When the natural habitat is transformed into AeroSystems, the composition and abundance of amphibian species are low since the animals do not have access to the necessary conditions to properly develop[15,16]. Abiotic factors, such as temperature, photoperiod, and water bodies, influence amphibian larvae and physiological mechanisms related to growth and differentiation[16]. Some species can adapt to these changes better than others. An example is a species of amphibians that lay their eggs in lentic water bodies and whose larvae develop without parental care. Habitat fragmentation significantly increases the vulnerability of amphibians to pesticide exposure. One example of this is the construction of roads that divide their habitats, forcing amphibians to traverse these barriers and consequently exposing them to higher quantities of chemical compounds, such as copper, lead, or petroleum hydrocarbons as they move between their terrestrial and aquatic environments[18].

Pesticides and other agrochemicals originate from human activity or agricultural farming[6]. Agrochemicals are applied in vast quantities of agriculture and urban areas worldwide[13,19]. Many pesticides and other agrochemical products are increasingly used worldwide, often in combination[20]. The continuous flux of crop rotation, land use, differences in chemical use, variable formulations, and application rates make it difficult to characterize and attribute causes and effects in field studies[20]. Pesticides appear in amphibian habitats from diverse sources. Several studies have been conducted *in vivo* and *in vitro* on amphibians, indicating that these compounds adversely impact population decline and health[21]. In areas where agricultural pesticides are heavily used, it is common to observe an exponential decay in amphibian populations [21,22].

One Health is defined as a collaborative, multisectoral, and transdisciplinary approach to sustainably balance and optimize the health of people, animals, and ecosystems[23]. Pollution is a problem under One Health since it affects the three with harmful effects[23]. This study aims to establish a link between pesticide and amphibian population decline, and to propose mitigation strategies for the future.

2. Exposure of amphibians to pesticides

Amphibians' life cycle includes aquatic and terrestrial phases (Figure 1) and migrations to and from spawning waters. Therefore, they can be exposed to pesticides in aquatic and terrestrial environments[20].

Pesticides can be found within the environment through multiple channels. They can disperse into the atmosphere (diffusion into the upper layers, deposition into the water, deposition into the soil), water systems (evaporation into the atmosphere, entry into groundwater, entry into bottom sediment, absorption by algae and plants, absorption by micro- and microorganisms) and soil (evaporation into the atmosphere, entrainment by surface drainage drains, infiltration into groundwater, accumulation in plants, accumulation by soil micro- and macroorganisms)[14,24]. Another route is over the trophic connections, which can be through the entry through food facilities, the consumption of amphibians by predators, or the Human consumption of amphibians for food purposes [11,14,24].

Many toads and other amphibians lay their eggs in

(A) (B)

Figure 1. A: a common toad (*Bufo bufo* [Linnaeus, 1758]), and B: a Bosca's newt (*Lissotriton boscai* [Lataste, 1879]) (Photo: Andreia Garcês).

water, where they develop as tadpoles or larvae[2]. Therefore, eggs, larvae, and tadpoles can be exposed to pesticides in water[2,22]. Moreover, the sediment may have pesticide concentrations 1000x higher than in water. In addition, amphibians can access sediment contaminants by ingestion, in the case of tadpoles and larvae, and cutaneous absorption, while hibernating in sediments, in the case of adults[2,25]. Adults and juveniles can be exposed to soil through direct aerial sprays, drift, and dermal absorption from the soil and plants[1].

Amphibian skin is highly permeable to gas, water, and ion transport in the environment[11]. It acts as a respiratory organ and regulates water intake in terrestrial and aquatic morphs[20].

Several researchers have investigated skin permeability in amphibians. Being permeable leads to faster cutaneous absorption than other vertebrates, with faster negative consequences for individuals[20,22]. For example, Quarants et al.[2] have measured the percutaneous passage of mannitol, antipyrine, atrazine, paraquat, and glyphosate molecules from amphibians and mammals. They used the skin of the *Rana esculenta* frog (amphibians) and the ear of swine (*Sus domesticus* mammals). They observed that the movement of these substances through the skin was notably more significant in frogs compared to pigs. The ration between percutaneous passage coefficient in frog / percutaneous passage in pig) was reported as 302 for atrazine, 120 for antipyrine, 66 for mannitol, 29 for paraquat, and 26 for glyphosate. These values indicated that the fluxes measured and, consequently, the rate of skin absorption were substantially higher in the skin of frogs in comparison to pigs.

In the terrestrial environment, amphibians are, therefore, susceptible to environmental stressors and vulnerable to desiccation and environmental pollutants since they depend on their skin to regulate the water balance (they get water through cutaneous absorption), ion transport, and breathing[11,12,20,26]. Toads, for example, uptake water by the pelvic area of the skin. Consequently, these amphibians may be exposed to water-soluble pesticides that run off vegetation or accumulate in small pools following precipitation[20]. After absorption, these compounds move to the venous circulation through the lymph canals to other parts of the body, with adverse consequences[11]. Regarding the absorption of pesticides, 83% can be absorbed through the dorsal or ventral skin, and 46% through the legs[20]. Frogs also shed their skin every 2 weeks and eat it for nutrition, meaning that the digestive tract probably absorbs any pesticide residues left on the skin[2,20].

Exposures typically follow a seasonal pattern and increase with each instance of application[2]. Figure 2 indicates how amphibians can be exposed to pesticides through dermal contact (direct contact with pesticide, contaminated water, contaminated plants), ingestion of contaminated water, or consumption of contaminated food. After this, compounds acummulated in amphoions can then accumulate in predators (e.g., birds of prey) that feed birds or even humans that consume amphoions [11,20].

When pesticides are introduced into the environment (Figure 2), the process can be beneficial or detrimental. Various soil and weather conditions, along with different handling methods, can either encourage or hinder each procedure. Grasping these processes can assist pesticide applicators in guaranteeing that their applications are both efficient and environmentally friendly[24].

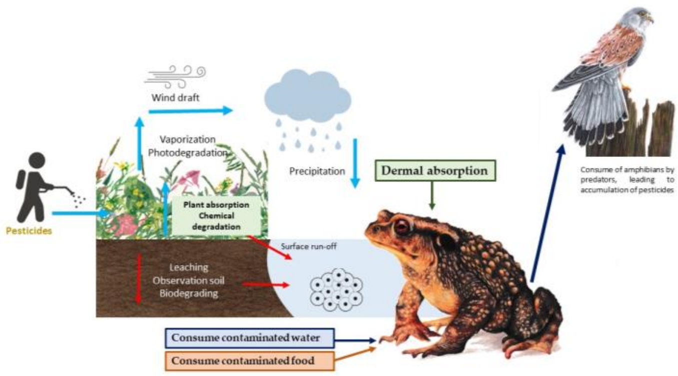

Figure 2. Pesticides dispersion methods (Illustration: Andreia Garcês).

The process involves adsorption, volatilization, runoff, and leaching. Adsorption is a critical process in the interaction between pesticides and soil, as it involves the binding of these chemicals to soil particles. Soils high in organic matter or clay are more absorptive than coarse, sandy soils. Moist soils generally have a reduced ability to absorb pesticides compared to dry soils because water molecules contend for the same attachment sites as the pesticides. For instance, paraquat or glyphosate are pesticides that exhibit strong binding with soil, while certain others bind weakly and can easily be released back into the soil solution[24]. Volatilization involves the transformation of a solid or liquid pesticide into a gas. Pesticides with higher vapor pressures tend to be more volatile. Various environmental factors, like elevated temperatures, low humidity, saturated soils, and air circulation, can intensify this process. When pesticides are present in vapor form and drift in the atmosphere, they have the potential to impact non-target species[24]. Runoff is a process where pesticides are carried by water in movement over the soil that is not observed or bounding to eroding soil. This phenomenon will depend on various factors, such as rate of an area, erodibility, texture and humidity of the soil, present of vegetation, volume of rainfall, and irrigation. Over-irrigation and heavy rains can lead to excessive runoff, especially after the application of the pesticides [24]. Finally, leaching refers to the pesticide's migration through the soil rather than across its surface which depends on the pesticide's specific chemical and physical characteristics. Pesticides that strongly attach to soil particles through adsorption are less prone to leaching, while those that readily dissolve in water can move along with the water. Soil attributes like texture, organic content, and permeability can impact the process of leaching[24].

3. Ecotoxicity studies in amphibians

Several entities, both public and private, play a crucial

role in assessing whether pesticides pose hazards to the environment and public health. In the United States, the United States Environmental Protection Agency (EPA) is a federal government responsible for environmental protection concerns[27]. This agency conducts environmental assessment, research, and education, protecting the population and the environment from significant health risks. In the European Union (EU), pesticides are regulated by Regulation No 1107/2009 on Plant Protection Products (PP) in cooperation with other EU Regulations and Directives provided by European Commission, European Food Safety Authority (EFSA), and European Chemical Agency (ECHA). All these agencies cooperate between themselves.

General ecological risk assessment methods use an organism to monitor contaminants and imply possible effects to biota or sources of toxins to humans. One of the major difficulties in this study is the choice of test organisms. Standard test animals used in ecotoxicology include earthworms, fish, alga, avian species, and rodents[28]. The Environmental Risk Assessment (ERA) assesses potential environmental harm caused by a substance, activity or natural occurrence, including compounds like pesticides [29].

Amphibian species have historically been utilized as models primarily in studies related to embryonic development and cell biology[28], with a limited focus on toxicology studies. In recent years, amphibians have increasingly attracted the attention of ecotoxicologists. In the 1990s, when amphibians declined and the appeal of malformations started, it led to a huge increase in ecotoxicological studies of amphibians [22,28]

Until a short while ago, Amphibians and reptiles were not directly included in the evaluation of pesticides' ecological risk assessment (ERA). This omission occurred because it was assumed that the assessments conducted on other vertebrate groups applied equally to these two classes. However, in 2013, the European Union introduced the first two regulations that specifically incorporated amphibians and reptiles into the pesticide ERA process[29,30]. Furthermore, in 2018, EFSA (European Food Safety Authority) released a scientific opinion that reviewed the current state of scientific knowledge regarding pesticide ERA for amphibians and reptiles. This opinion will serve as the foundation for future documentation in pesticide authorization and regulation, with the aim of minimizing the need for additional testing.[29,30].

4. Impact of pesticide exposure on the amphibian population

There are several studies *in vivo* and *in vitro* regarding the impact of pesticides on amphibians. Although *in vitro* studies are very useful, they only partially show the full effect of these agents. These compounds are usually studied isolated or in a combination of two, under strict laboratory conditions. In the natural habitat, it is not so simple to predict the effects of pesticides in the organism[30,31]. Diverse factors strongly influence the bioavailability of these chemicals as differing physicochemical properties of the chemical, the abiotic/biotic features of the environment, and the intrinsic physiologies of the exposed organisms[31]. Factors such as temperature or pH, can impact the toxicity of pesticides, making them more or less toxic[1,11,32]. Organophosphates, synthetic pyrethroids, carbamates, and chlorinated hydrocarbons lead to their hydrolysis when mixed in water with a PH greater than 7.0 [33].

Other two important properties of pesticides that allow their accumulation in sediment and water are hydrophobicity and persistence (e.g., DDT). Pesticides have had the potential to accumulate in sediment and aquatic biota if water solubility is less than 1 milligram per liter (mg/L) or an octanol-water partition coefficient (Kow - lipid solubility) greater than 1,000, and a soil half-life greater than 30 days[33]. Compounds that possess low water solubility and exhibit high persistence are easier to find. Pesticides are seldom detected in water, and sediments tend to have greater water solubility and shorter soil half-lives. Pesticides with moderate detection frequencies (like mirex and endrin) fall in the middle ground regarding their hydrophobicity and persistence[34]. This structural-activity relationship is employed to assess whether pesticides have the potential to accumulate in sediment and aquatic organisms34. In contrast, modern pesticides generally have relatively high water solubility and shorter soil half-lives, making them less inclined to accumulate in these environments. Nevertheless, some presently used pesticides, which fall in the intermediate range for both hydrophobicity and persistence (e.g., Chlorpyrifos, Pendimethalin), may still be detectable if examined in sediment and aquatic organisms, albeit with lower detection frequencies compared to highly hydrophobic and persistent pesticides like DDT[34].

Moreover, pesticides suffer degradation in the environment. There are three types of pesticide degradation, namely microbial (in soil some species of fungi, bacteria, and other microorganisms can use pesticides as food source), chemical (e.g. hydrolysis), and photodegradation (breakdown of pesticides by light, on the foliage, on the surface of the soil, and air) [35].

There are several descriptions of toxicity in the natural habitat of amphibians with elevated mortality rates. For instance, endosulfan, dacthal, and chlorthalonil were detected in the mountain soils of Costa Rica. These compounds are incredibly toxic to amphibians and many amphibian species in this region, including *Incilius periglenes* and *Atelopus varius*, have gone extinct.[15].

In wetlands near agricultural sites in Iowa, the USA, pesticides have been detected in water, sediment, and animal tissues. Thirty-two pesticides were found in the water, mainly atrazine, metolachlor, and glyphosate. The mean concentration of atrazine in the water was 0.2 ppb, sufficiently high to cause reproductive abnormalities. Fourteen pesticides were detected in the sediment, most prometon and metalalaxyl. About 17 pesticides have been

found in *Pseudacris maculata* and *Lithobates pipiens* frog tissue. The most frequently detected were fluoxastrobin, pyraclostrobin, metolachlor, and bifenthrin. Maximum whole frog concentrations were 470 ppb w weight[36].

In agricultural wetlands of Cerro Gordo and Worth counties, Iowa, the USA, 72 northern leopard frogs, *Lithobates pipiens*, were studied during 2016 concerning the presence of pesticides in the liver and gonads. There was detected the presence of fenbuconazole, tebuconazole, bifenthrin, and p, p'-DDE[37].

In California, amphibian declines were first noted in 1970. The fall of amphibians was associated with the application of DDT and organochlorines in the Central Valley that was blown by the wind up to the Sierra Nevada, leading to mortality of 95% in species, such as frogs *Rana muscosa*, *Rana sierrae*, and *Rana boylii* (Baird, 1854)[38]. Chlorpyrifos (92-276 LD50 oral, 2,000 LD50 dermal mg/Kg) is concentrated in animal tissues and sediments. Sediments taken from mountain lakes in California have been discovered to contain concentrations as high as 161ppb[38]. In San Joaquin Valley in 1995 around 5.9 million kg of agrochemicals were applied to that area[39]. Chlorpyrifos and endosulfan concentrations ranging from 4-12 ng/liter have been detected in lakes. In the tissues of *Pseudacris regilla*, chlorpyrifos concentrations of 13 ng/g and endosulfan concentrations of 22 ng/g were observed. Additionally, sediment samples contained chlorpyrifos at concentrations of 161 mg/kg and endosulfan at concentrations of 48.7 mg/kg[38]. Endosulfan poses a danger to amphibians in the mountains of the Sierra Nevada. Low levels of cholinesterase have been reported. Nearly, 60-80% of *Pseudacris regilla* populations showed a 50% reduction in cholinesterase enzyme activity, compared to 9-17% in regions that were not affected[40]. Furthermore, 86% of certain populations had measurable levels of endosulfan, and 40% had detectable residues of 4,4′-dichlorodiphenyldichloroethylene, 4,4′-DDT, and 2,4′-DDT40[40]. The decline of *Rana muscosa*, has been associated with great exposure to chlorpyrifos, endosulfan, chlordane, nonachlor, dacthal, and DDE in those areas[41]. Pesticide residues have also been detected in the Cascades, where the *Rana cascadae* is the species most affected. The most frequently discovered residues were endosulfan, chlorpyrifos, dacthal, PCB, chlordane, and nonachlor [39]

A study conducted in Quebec, Canada, revealed that hind limb deformities were commonly observed in Bullfrogs (*Lithobates catesbeianus*), Green Frogs (Lithobates clamitans), Northern Leopard Frog (*Lithobates pipiens*), and American Toads (Anaxyrus americanus). The deformity rates tended to be higher in agricultural areas, suggesting that herbicides and pesticides are the likely causes[6].

Two frog species, *Leptodactylus latinasus* and *Leptodactylus latrans* inhabit the agricultural areas of Pampa, Argentina, in close proximity to crops. Pesticide residues were observed in the muscle and kidney tissues of 64 animals from both species[42]. A total of 20 different pesticides were found in the tissues, such as chlorpyrifos-methyl, pirimiphos-methyl, and acetochlor. In general, one or more pesticide residues (up to 12 in a single frog) were identified in 40-57% of *L. latrans*. *L. latinasus* had presented more pesticide detections than *L. latrans*[42].

In south Germany, a study on common toads (*Bufo bufo,* in pesticide-intensive viticultural, showed that the toads from more contaminated ponds (more than 22 different pesticides) had an elevated fecundity (more eggs), but reduced fertilization rates (fewer hatching tadpoles) as well as lower survival rates and reduced size of the tadpoles[43].

Two regions of South Africa, the Kruger National Park and Ndumo Game Reserve, were examined for organochlorine pesticide accumulation in *Pyxicehaplus edulis, Hildebrandtia ornata, Sclerophrys garmani, Chiromantis xerampelina, Ptychadena anchietae,* and *Xenopus muelleri*. Of the 22 analyzed organochlorine pesticide, 12 DDT, DDD, and chlordane were detected in samples[44].

Table 1 provides some examples of environmentally relevant pesticides and their effects on different amphibian species in different regions.

Table 1. Examples of pesticides relevant to the environment and impacts on different amphibian species in different regions (LD_{50} values are mg/kg)

Compound	Concentration environment (ppb)	LD_{50} oral	LD^{50} dermal	Specie	Country	Effect	Ref.
Atrazine	0.1 -100	1,869	>3,100	*Rana pipiens* *Xenopus laevis*	USA	10–92% of males show gonadal abnormalities, desmacularization, hermaphroditism	45,46
	100			*Rana pipiens*	USA	Retarded gonadal development, hermaphroditism	47
Endosulfan	10-1700	160	359	*Rana dalmatina*	Italy	Mortality	48
Endosulfan	60			*Rana pipiens*	USA	97% mortality	49
Chlorpyrifos	0.33 -3.96	92-276	2,000	*Rana boylii*	USA	Mortality	38
Cypermethrin	0.6	250	2,000	*Rana arvalis*	Germany	reduced hatching rates, prolonged the time needed for metamorphosis, and induced deformities and irregularities in behavior.	50
Propiconazole	74	1,517	>4,000	*Bufo cognatus*	USA	40% mortality	51
Pyraclostrobin	150	>500	>4,000	*Bufo cognatus*	USA	100% mortality	51
Chlorothalonil	164	>10,000	>10,000	*Rana sphenocephala*	USA	86% mortality	52
Imidacloprid	240	tech 450	>5,000	*Rana nigromaculata*	France	Genetic damage	53
				Acris crepitans	USA	Mortality	54
Glyphosate	2000	>5,000	>5,000	*Hyla versicolor*	USA	80% mortality	55

5. Pesticide toxicity to amphibians

Roundup (broad-spectrum glyphosate-based herbicide) in larval species can cause 96% mortality and in adult toxic than the active ingredient[56]. An example is the combination of herbicides (atrazine and metolachlor) and insecticide (chlorpyrifos), when applied in combination with *Hyla versicolor* tadpoles, leading to 100% mortality in laboratory[57].

Pesticide mixtures can have even more harmful effects [2,21,45]. Worldwide, various pesticides have been used together, raising concerns about the potential long-term exposure of amphibians and other animals to low doses of these pesticide combinations, which can have adverse health effects that are challenging to assess[58]. The identification of these synergistic interactions between the different compounds in order to determine the cumulative risk assessment is necessary, but it can sometimes be difficult to determine. These compounds have the ability to interact with each other according to the toxicokinetic and toxicodynamic, such as active principle, dose, chemical family, and targeted organs[59]. These interactions can lead to unpredictable effects, as some or all of the components present in a mixture can influence each other's chemical toxicity. Currently, the most frequently identified synergistic combinations involve cholinesterase inhibiting insecticides, triazole fungicides, triazine herbicides, and pyrethroid insecticides60. The opposite reaction can also happen, in which combined exposure of two or more pesticides together results in reduced toxicity compared to the individual chemicals (antagonism). For example, binary mixtures of OPs produced less than additive effects on voltage-gated calcium channels[59].

However, it's important to note that the data available from real-life exposure scenarios, particularly at environmentally or dietary relevant concentrations of pesticides, do not provide sufficient information to fully understand the actual impact of these interactions on the health of both animals and humans. Future studies should focus on understanding the danger assessment of pesticide mixtures at realistic doses, model their cumulative effects, identify the groups of pesticides that are likely to cause synergistic interactions, and develop new sophisticated experimental methodologies for testing pesticide mixtures[58].

Moreover, pesticides also interact with other stressors, such as environmental temperature or UV radiation [20].

Several studies have shown that pesticides may increase disease progression, transmission, and mortality. In addition, they may modify cutaneous microbiomes by improving the successful colonization of pathogenic microorganisms[12,21,32]. Moreover, pesticides can inhibit cholinesterase production, accumulating acetylcholine in the postsynaptic membrane, resulting in Parkinson's disease and leading to the animal's death[11].

There is a great variability of pollutants and a significant deviation among amphibians, so animals could be affected differently[21]. They are generally susceptible to most pesticides, especially insecticides, herbicides, and fungicides[13,] and may be affected by acute or chronic toxicity with several consequences[6,11,21]. Acute toxicity is short-term toxicity caused by a single exposure. It is measured as LD_{50} (lethal dose 50), the concentration of compound that can kill 50% of animals in the test population. This exposure can result in severe skin, eye, mouth and lung injury and death[11].

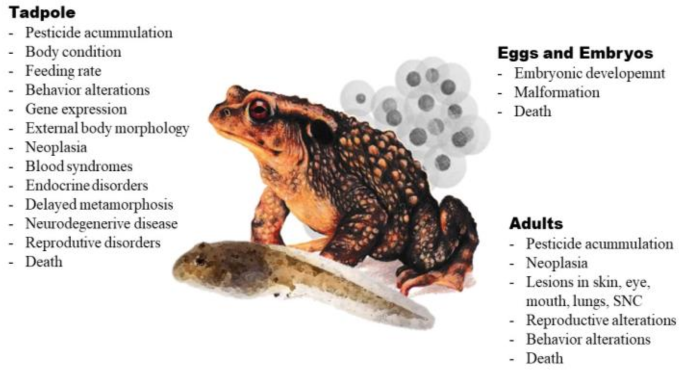

Figure 3. Potential pesticide effects in different life stages of amphibians (Illustration: Andreia Garcês).

Low concentrations of pesticides in the environment, which may vary in terms of concentration, timing, and frequency, can have indirect effects that ultimately prove harmful to amphibian populations[61]. While many studies have shown that insecticide and other pesticide concentrations in natural habitats are lower than concentrations that directly cause lethal effects on amphibians in laboratory settings[61], it is evident that amphibian populations are still declining, largely due to sublethal effects[62].

In cases of chronic intoxication, exposure is at low doses. Generally, juveniles are more sensitive than adults and can die[2]. In addition, chronic intoxication may be responsible for the development of neurodegenerative diseases, growth reduction, delayed metamorphosis, congenital defects, formation of benign or malignant tumors, blood syndromes, genetic changes, increased predation, deformities, endocrine disorders, and low reproductive activity (Figure 3)[2,11].

5.1. Reproductive alterations

Various pesticides and related chemical compounds may act as hormonal or endocrine disruptors (EDCs), interfering with hormonal regulation, chemical messengers, and their metabolic pathways[6,45,64]. These compounds affect embryos, larvae, and adults[63,64]. Studies have indicated that EDCs at low concentrations can lead to abnormal sexual development, abnormal sex ratios, unusual behavior, alterations in immune or neurological function, malformations, disruption of the thyroid and other endocrine organs, and neoplasia[6,45,64].

Some studies have shown that the exposure of an adult male to EDCs can lead to chemical castration and complete

feminization. In Hayes et al.'s (2010) study, adult male African clawed frogs (*Xenopus laevis*, Daudin, 1802) were exposed to atrazine over 2-3 years. Atrazine, a chemical with an LD50 of 1,869 mg/Kg when administered orally and 3,100 mg/Kg when administered dermally, was used at a concentration of 2.5 parts per billion (ppb) and dissolved in ethanol for the exposure[65]. Animals that were exposed to atrazine experienced a range of adverse effects, including reduced testosterone levels, smaller breeding glands, altered masculine characteristics, feminized laryngeal development, diminished mating behavior, lower sperm production, and reduced fertility. Notably, the study's results revealed that 10% of these animals underwent a transformation into functional females, which then mated with males that hadn't been exposed to atrazine, resulting in the production of fertilized eggs[65].

A study conducted in a viticultural area in the Palatinate region of Southwest Germany, focusing on common toads (*Bufo bufo*), revealed that in ponds with higher contamination levels, where a mixture of more than 10 different types of pesticides was present, there were an elevated fecundity (more eggs) but reduced fertilization rates (fewer hatching tadpoles), lower survival rates of tadpoles and decreased size in Gosner stage 25[43].

5.2. Biometry and behavior alterations

The exposition of CuSO4 and Bordeaux mixture (fungicides) on the early stages of development of *Xenopus laevis* in high concentrations induced spontaneous maturation and affected tadpoles' biometry leading to animals with larger heads or body[66,67].

Pesticides have also been shown to modify predation-prey interactions[68]. In amphibians, predation can lead to higher sublethal pesticides becoming lethal. This can occur through direct mortality caused by pesticides or indirectly through reduced predator recognition, avoidance, or growth[21,68]. Insecticides, in particular, seem to increase rates of abnormal swimming, leading to a reduced escape response to predator attacks[69]. When tadpoles were exposed to components such as organochlorine endosulfan[70] or imidacloprid[71], they started swirling rapidly and presenting abnormal swimming patterns.

Exposition during 28 days of *Limnonectus limnocharis* tadpoles to malathion (diethyl [(dimethoxy phosphino thioyl] butanediote), lead to a decrease in tadpole survival from 20 to 6 tadpoles, decrease in growth, and decreased in food consumption (0.067 mg.g[-1].d[-1] to 0.0075 mg.g[-1].d[-1]) in the higher doses of exposition (3000 mug L[-1])[72].

5.3. Alterations in the immune system and susceptibility to disease

The skin of amphibians also plays a vital role in regulating animal health through the production of antioxidants and antimicrobial peptides as part of the innate immune system[12]. Additionally, the skin has populations of bacteria that persist in antimicrobial mucosa that can inhibit pathogen colonization skin infection. Any changes in this microbiome, such as chemical exposure, can lead to a higher prevalence of infections and mortality [12].

As mentioned, pesticides can lower the immune response of amphibians. Studies have shown that exposure to pesticides has been linked to heightened vulnerability to infections, increased levels of pathological conditions, and greater parasite numbers in amphibian populations[12,73]. The exposure to these compounds alters amphibians' resistance mechanisms by disrupting immune function, reducing leucocyte counts, or decreasing cholinesterase activity[73]. Therefore, these animals are more susceptible to diseases such as the fungal pathogen *Batrachochytrium dendrobatidis* (Bd)[74]. The relation between pesticides and Bd is not very well understood. However, some studies have shown that pesticide exposure may alter the dynamics of Bd and amphibian hosts[12,75]. Some authors state that since Bd is confined to the skin of infected animals, the immune defenses of the skin, including antimicrobial peptides, that should protect hosts from infection might be compromised by exposure to contaminants[12,75].

In a laboratory study, cercariae of the trematode Echinostoma trivolvis (parasite), snails (Planorbella trivolvis, first host), and green frog tadpoles (Rana clamitans second host) were exposed to 201.0 lg/L atrazine, 3700.0 lg/L glyphosate, 33.5 lg/L carbaryl, and 9.6 lg/L malathion[76]. Sublethal exposure of *R. clamitans* to these pesticides led to their susceptibility to infection by *E. trivolvis*, since their immune system was weaker. This was assessed by the percentage of cercariae that encysted in the amphibians. Therefore, the exposure to environmentally realistic levels of pesticides will lead to higher amphibian trematode infections[76].

5.4. Teratogenic effects

The exposure to pesticides can lead to the development of malformations in amphibians during fetal development. It has been demonstrated that malformation rates are much higher in water sources contaminated by pesticides, up to 60%[77].

Teratology is the research of congenital malformations of embryos and their causes[77,78]. These deformities may encompass structural or anatomical irregularities within the organs. A wide range of factors can be linked to the occurrence of such deformities, including genetic factors, environmental factors, or multifactorial influences[79,80]. There are some reports of teratogenic effects in amphibian's species, some observed *in vitro* and other *in vivo*, in different stages of the life cycle. Table 2 shows some examples.

6. Pesticides and climate change

Numerous research studies have explored how climate change impacts the harmful effects of chemicals like pesticides on aquatic organisms. Some of these studies

Table 2. Examples of pesticides and their teratogenic effects

Specie	Chemical compound	Teratogenic effect	Ref.
Microhyla ornata	Benzene hexachloride	Growth of body cavities, alteration in blood circulation, decreased pigmentation, body axis curving, retarded growth, blistering	81
Rana kl. Esculenta	Heptachlor	Alterations epidermis, damaged mitochondria	82
Bufo arenarum	Dichlorodiphenyltric hloroethane	Body torsion, edema, , neurological abnormalities, reduced weight, defective gills, decreased metamorphosis time	83
	Dieldrin	Exogastrulation, body twisting, alterations in the pigmentation, smaller larvae, anomalies in the gills, irregular swimming, hyperexcitability, alterations in the metamorphosis time	83
	Lindane	Body torsion, hyperactivity, progressive hydropathy, decreased metamorphosis time, erratic swimming, defective gills, reduced weight, caudal fin flexion	83
	Azinphos methyl	Reduced growth, notochord twisting, irregular pigmentation, malformations in the gut and gills, irregular swimming	83
	Malathion	Body bending, alteration in the movements, poor pigmentation, notochord curvature, hemorrhage, edema, atypical swimming, tail folding, smaller gills, reduced body length	83
	Parathion	Reduced body length, edema, anomalies in pigmentation, notochord twisting, anomalies in the gut and gills, hemorrhage, atypical swimming	83
	Carbaryl	Reduced growth length, atypical swimming, anomalies in the pigmentation, anomalies in the gut and gills, notochord twisting	83
Rana temporaria	Dieldrin	Reduced weight, deformed muzzle, notochord alteration, hyperactivity	84
	Oxamyl	Body bending, reduced development	85
Limnodynastes tasmaniensis	Dieldrin	Abnormal otolith, cephalic pigmentation	86
Ambystoma barbouri	Endosulfan and octylphenol	Atypical hatching, respiratory alterations, reduced growth rates, larval mortality, limb malformations	76
	Carbaryl	Atypical hatching, respiratory distress, larval mortality, reduced growth rates, limb malformations	76
Rana catesbeiana, Rana sylvatica, Bufo americanus	Toxaphene	Irregular swimming, atypical posture, balance disturbed,	87
Rana catesbeiana	Malathion	Malformed gills, paralysis, alteration in the coordination and equilibrium, hemorrhage	88
Xenopus laevis	Malathion	Abnormal neuromuscular, reduced growth size, altered swimming, atypical pigmentation, notochordal and tail curvature, anomalies in the gut	89
	Carbaryl	Abnormal tail curvature, alterations of the notochord, skeletal muscle lesions	90
	Chloranil; dichlone; nabam	Altered cephalic development, reduced growth size	91
	Amitraz	Growth delay, flexure of the notochord or tail, face, heart and abdomen edemas	92
Rana perezi	Methyl parathion	Scoliosis, tail folding, notochord bending	93
	Carbamate ZZ-Aphox	Lesions in gill, liver, gall bladder, heart, notochord	93
	Pirimicar	Enlarged length size, tail torsion, limb deformity	94
Gastrophryne olivacea, Bufo bufo gargarizans, Ambystoma mexicanum, Rana boylii	Chlorpyrifos	Shrivelled fins, tail curvature, behavioral changes, tail deformities and head edema, malformed notochord, tadpole mortality, defects of neuromuscular activity	95

Note: Adapted from [77]

indicate that climate change may elevate the toxicity of contaminants, while others suggest that contaminants may reduce tolerance to extreme temperatures[94]. Amphibians may be more sensitive to interactions between climate change and contaminants due to their skin permeability, offering little resistance to water loss through evaporation or uptake of pollutants[94]. Furthermore, amphibians are ectotherms and are more susceptible to temperature changes[94]. For example, rising temperatures increased atrazine's toxicity in catfish[95] and intensified the adverse effects of carbaryl, endosulfan, and esfenvalerate on amphibians[96]. Temperature increases due to global climate change might also accelerate embryonic and larval development, reducing the amount of the aquatic stages of amphibians and, thus, the duration of their contaminant exposure. At the same time, this increase could increase pesticides' chemical toxicity[95].

Moreover, it is important to refer to climate change in the circulation of pesticides. The increase in temperatures can have an impact on the increase in temperature of reservoirs, decrease in precipitation and shallowing of reservoirs, and decrease in soil moisture [94].

7. Amphibian adaptations against pesticides

The global change caused by human activities pose a significant dilemma for organisms, as they must either move to regions with more favorable environments or adapt to the new conditions[96].

In a study on wood frog (*Lithobates sylvaticus*) population, it was found that those frogs live close to agriculture were more resistant to chlorpyrifos, but not to Roundup. Frogs closer to agriculture fields with chlorpyrifos had higher survival than populations farther from agriculture[96]. Also, no evidence was found that this resistance carried a performance cost when facing competition and the fear of predation[96]. This is the example of a vertebrate species acquiring pesticide resistance through a process denominated phenotypic plasticity, in which the expression of some genes changes in response to environmental pressure. It occurred in only one generation, not involving the genes itself [97].

Another study compared the resistance of amphibian populations from treated and untreated reference sites, using DDT. The population from the uncontaminated site was very sensitive. Still, there was no clear mortality pattern for sites that were sprayed directly versus sites that probably experienced indirect exposure[96]. Research on carbaryl reveals that resistance to pesticides can differ among amphibian species, populations, and individual organisms. Laboratory tests showed that *Oophaga sylvatica* embryos and hatchlings living far from farmers' fields were not pesticide-resistant but could quickly become tolerant when exposed to low levels of pesticides[98,99].

8. Conclusions

Amphibians are a highly diversified animal group that plays an important and varied role in ecosystems. Aggressions affecting their number and biodiversity are often only identified in the medium term when the consequences are irreversible.

Human activities could damage ecosystems, the biodiversity of species, and the health of the environment in which he is involved. One example is the use of contaminants, which are harmful with long-lasting effects. Moreover, combining chemicals or associations with other environmental factors may be even more harmful and have consequences for survival that are tough to predict.

Studies are still rare and may not reflect the high variability of pollutant exposure responses across species, the pollutant type, and environmental conditions. Therefore, it is imperative to study amphibian populations under natural conditions to assess human activities' impact on biodiversity and survival. Studies like this will help provide EFSA with information relating to assessing the risk to amphibians posed by pesticide exposure. Also, EPA usually does not require amphibian toxicity tests for pesticide registration, and with these studies, they could start including this group of animals. This could be the only way to identify threats and implement effective and timely measures to avoid irreversible harm to the survival of species, damage to ecosystems, and ultimately, to animals and humans under the One Health concept.

Declarations

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Andreia Garcês and Isabel Pires conceptualized the study. All authors developed the methodology collaboratively, ensuring a comprehensive approach to the investigation. The entire team carried out the formal analysis and investigation, collectively contributed to the data collection and analysis process. All authors participated in the writing of the original draft of the manuscript. All authors checked and approved the final version of the manuscript for publication in the present journal.

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